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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 15525.12-L; 18241.1-L	2. GOVT ACCESSION NO. AD-A107 868	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) The Physiology, Biochemistry and Genetics of Survival of Bacteria Subjected to Environmental Stress	5. TYPE OF REPORT & PERIOD COVERED Final Report: 1 Jun 78 - 31 Aug 81	
7. AUTHOR(s) P. H. Calcott	6. PERFORMING ORG. REPORT NUMBER	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Wright State University Dayton, OH 45435	8. CONTRACT OR GRANT NUMBER(s) DAAG29 78 G 0084; DAAG29 81 K 0103	
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Research Office Post Office Box 12211 Research Triangle Park, NC 27709	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	12. REPORT DATE Nov 81	
	13. NUMBER OF PAGES 46	
	15. SECURITY CLASS. (of this report) Unclassified	
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) NA		
18. SUPPLEMENTARY NOTES The view, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This report contains the results of an investigation on the microbial physiology, biochemistry and genetics. Since several basic questions and approaches were pursued in these studies, the report is divided into six parts: 1) the effects of stress on metabolic activity of bacteria; 2) the effects of stress on genetic stability; 3) the relationship of cell composition to survival potential; 4) starvation and substrate-accelerated death in bacteria; 5) cryoprotection studies; 6) survival and enumeration of bacteria in natural systems.		

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FINAL REPORT FOR TWO RESEARCH GRANTS TO

Dr. P. H. Calcott

Department of Biological Sciences
Wright State University
Dayton, Ohio 45435

Grant No. DAAG 29-78-G-0084

"The effect of a variety of stresses on the persistence, detection and enumeration of bacteria of public health significance with particular reference to arctic and frozen habitats"

and

Grant No. DAAG 29-81-K-0103

"The physiology, biochemistry and genetics of survival of bacteria subjected to environmental stress"

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This report constitutes the final and technical report by Dr. Peter H. Calcott for the two above named grants. The first grant covered the 3 year period 1st June 1978 to 31st May 1981 while the second covered a three month period 1st June 1981 to 31st August 1981. The second grant was terminated upon request of both Dr. P. H. Calcott and Wright State University since the former had been offered a new position in Central Research-Bioproduets, 1701, The Dow Chemical Company, Midland, Michigan 48640, and was unable to continue the work at the new locale; an alternative principal investigator was also not available at WSU to continue the studies.

These two grants, which both concerned the effects of stress on microbial physiology, biochemistry and genetics, will be reported as one. Since several basic questions and approaches were pursued in these studies the report will be divided into six parts.

- 1) The effects of stress on metabolic activity of bacteria.
- 2) The effects of stress on genetic stability.
- 3) The relationship of cell composition to survival potential.
- 4) Starvation and substrate-accelerated death in bacteria.
- 5) Cryoprotection studies.
- 6) Survival and enumeration of bacteria in natural systems.

1. The Effects of Stress on Metabolic Activity of Bacteria

a. Active transport

Personnel: S. Steenbergen, B.S.; D. Williams; R. Petty, B.S. M.S.; and P. H. Calcott P.I.

Publications: 2, O.J.S. abstract, FEMS letter (see Appendix A).

This study, started before the grant was awarded, was continued, published as a paper and presented by Ms. S. Steenbergen. In addition, Ms. D. Williams continued the study and examined the effect of stress of two specific transport systems for arabinose in E. coli.

Wild-type strains of E. coli are capable of transporting arabinose by a membrane-bound permease or by a periplasmic binding protein system. Using mutants defective in each of the mechanisms, Williams concluded that the membrane bound transport system (permease) was inactivated in a similar fashion to that of bulk amino acid transport as studied by Steenbergen. This suggested that the amino acid transport system studied previously was probably permease-driven. The periplasmic binding protein system was more sensitive to stress than the permease but was still able to be repaired. These observations have not been published.

b. Protein synthesis

Personnel: B. Ghani B.S. M.S.; P. Calcott, P.I.

Publications: 3, O.J.S. Abstract, FEMS Letter, M.S. Thesis
(Appendix A)

Mr. Ghani examined protein synthesis in frozen-thawed E. coli for his M.S. Thesis. The results were presented at a meeting and are summarized in the abstract of his thesis. A small portion of his study was published. The remainder is to be written up in a paper.

c. Induction of operons

Personnel: M. Thomas, P. H. Calcott, P.I.

Publications: 0

Frozen and thawed E. coli show a phenomenon called "metabolic injury". This is manifest when the stressed organisms are challenged with a nutrient rich (nutrient agar) and a nutrient poor (mineral salts and glycerol agar) medium with the plating efficiency being high and low respectively. This phenomenon reported many times over the last twenty years has never been fully explained.^{1,2} The factor(s) present in the rich, but not the poor, medium has been sought and found many times. However, in some systems it is one or a family of amino acids while in others it is short peptides. This lack of consistency has led me to postulate that metabolic injury is really an example of disruption of cell control processes. Since small molecules such as cyclic AMP, cyclic GMP and guanosine tetraphosphate have been implicated in cell control processes, this has led me to determine whether the nucleotides, singly or in combination, could alleviate metabolic injury. Using a strain of E. coli used by others to study the phenomenon, we found that 1 mM cyclic GMP (G) or 1 mM guanosine tetraphosphate (T) but not cyclic AMP (A) in the plating medium could partially alleviate the phenomenon. Moreover, in combination, A+T, G+T, A+G or A+T+G, the phenomenon was abolished. Organisms when incubated in a single salts solution or nutrient broth at 37°C for 1 hour repaired the injury and did not exhibit the phenomenon. Organisms which showed metabolic injury (decreased ability to grow on a mineral salts agar medium) also induced the lac-operon at slower rates than unstressed cells only when challenged with lactose as inducer (not the gratuitous inducer, IPTG) in a salts medium with glycerol (not nutrient broth). Organisms allowed to repair 1 hr at 37°C before challenge behaved like unstressed organisms. When the induction medium (mineral salts + glycerol) was supplemented with 1 mM cyclic GMP or guanosine tetraphosphate, stressed organisms induced the

lac-operon at normal rates; 1 mM cyclic AMP did not alleviate the phenomenon. I suggest that metabolic injury is a phenomenon connected with the general control of cell metabolism. Stresses, such as freezing and thawing, disrupt the production and degradation of control nucleotides and other soluble intermediates. The "incorrect" levels of these nucleotides for that particular environment cause the organism to be unable to divide when challenged with an otherwise acceptable growth medium. There appears to be many similarities between metabolic injury and substrate accelerated death (Section 4).

d. Effect of cytoskeletal disruptive drugs on cell viability and metabolism

Personnel: B. Ghani, B.S. M.S.; R. Petty B.S. M.S.; K. Knisley B.S. M.S.; D. Morton B.S. M.S. Ph.D. (Dept. of Biol. Sci., WSU); P. H. Calcott, P.I.
Publications: 1, OJS Abstract (Appendix A)

E. coli can grow in the presence of very high concentrations of the eucaryotic cytoskeleton disruptive drugs, cytochalasin B, colchicine and vinblastin. However, stressed organisms (frozen and thawed in water) are susceptible to these drugs at concentrations much higher than for eucaryotes. Presumably the freezing process makes the cell wall and membrane permeable to the drugs. All three drugs can decrease viability of stress populations to that of saline frozen ones in a dose dependent manner. At concentrations which decrease viabilities, vinblastine inhibited RNA and protein synthesis by 90 and 40% respectively while colchicine inhibited these two processes by 80 and 65%; neither drug inhibited DNA synthesis by more than 5%. Cytochalasin B, while not inhibiting protein synthesis, inhibited DNA and RNA syntheses by 95 and 85% respectively; low concentrations of the drugs which did not affect viability were without effect on any macromolecular synthesis rate. Electron-microscopic examination of stained thin sections of the organism showed that the frozen cells possessed condensed nuclear regions while drug treated cells contained either very condensed single nuclear regions or condensed dispersed nuclei (dependent on the drug).

This study indicates that the cytoplasm and nucleus of this procaryote is possibly highly organized and shares similarities in organization with eucaryotes. Since these drugs are active not only against viability, and biochemical activities but also ultrastructural properties, it would suggest that a cytoskeleton is present in procaryotes that controls central processes.

This study is being written up as a paper for submission in the next six months.

e. Loss of mercuric ion resistance in *Pseudomonas aeruginosa*

Personnel: P. H. Calcott P.I.

Publications: 3, FEMS Letter, Applied and Environmental Microbiology paper, 2nd International Symposium on Microbial Ecology, Abstract (Appendix A)

This study was completed and published.

2. Effects of Stress on Genetic Stability

a. Mutagenic action of freezing

Personnel: A. M. Gargett B.S.; P. H. Calcott P.I.

Publication: 1, FEMS Letter (Appendix A)

b. Sensitivity of DNA repair deficient mutants of *E. coli*

Personnel: M. Thomas; P. H. Calcott P.I.

Publication: 1, FEMS Letter (Appendix A)

c. Sensitivity of DNA repair deficient mutants of *P. aeruginosa* and effect of an R-Plasmid

Personnel: D. Williams; P. H. Calcott P.I.

Publications: 2, Journal of General Microbiology, Miami Tissue Culture Association Abstract (Appendix A)

d. Plasmid curing

Personnel: L. Anderson; D. V. Wood B.S.; P. H. Calcott, P.I.

Publication: 1, 2nd International Conference on Microbial Ecology, Abstract (Appendix A)

Two plasmids (RP4 in *E. coli* and PPL1 in *P. aeruginosa*) can be cured not only by regular curing agents, heat, detergent, acridine orange, acriflavin, mitomycin C, pyocyanine but also by freezing and thawing. The freezing and thawing process, not the storage event is responsible. Even in the worst situation less than 10% of the population was cured of plasmid. This suggests that stresses such as freezing might play little role in curing natural populations of their plasmids unless the plasmid is relatively unstable, in which case the stress might play a significant role. These data are to be written up as a short note.

e. DNA damage and its repair

Personnel: J. C. Song B.S. M.S. (agr) M.S.; P. H. Calcott P.I.
Publications: 2, Miami Valley Tissue Culture Association Abstract
M.S. Thesis (Appendix A)

Mr. Song conducted a study to determine whether single strand breaks were introduced during freezing and thawing. This study was completed; he successfully defended his thesis and presented a poster session of his data.

3. The Relationship of Cell Composition to Survival Potential

There are two ways to approach this problem. Organism composition can be changed phenotypically by altering the cell's environment which results in altering the cell's physiology and composition. Alternatively, cell composition can be altered genetically. Both approaches have been utilized in these studies.

a. Chemostat study

Personnel: R. Petty B.S. M.S.; J. Runkle B.S. Ph.D. (Dept. Biol. Sci., WSU); P. H. Calcott P.I.
Publication: 1, FEMS Letter (Appendix A)

By growing E. coli in chemostat at four different growth rates (0.1, 0.2, 0.3, 0.5 h⁻¹) under carbon and ammonium limitations, we have obtained eight phenotypically different populations. They differ in response to survival on five different media (nutrient agar (NA), detergent supplemented NA, salt supplemented NA, Endo agar, MF-C agar) after slow or fast freezing and slow or fast thawing. In general, survival is highest in slowly grown versus rapidly grown, nitrogen versus carbon-limiting conditions. Survival is highest on nutrient agar, followed by salt supplemented agar; survival on the endo or MFC agars as well as detergent supplemented agar is very low. Organisms survive better when frozen at fast versus slow rates and thawed rapidly versus slow. Cell composition is affected by the growth environment. Lipid (see attached paper) and lipopolysaccharide (L.P.S.) compositions are affected. Under ammonium limitation the chain length of the L.P.S. is increased (measured by hexose, hexosamine, sedheptulose, pentose to KDO ratios). The precise relationship between cell composition and survival potential is under statistical analysis by Dr. J. Runkle and will be available in 3-6 months.

b. Plasmid mediated alteration in cryoresistance in *P. aeruginosa*

Personnel: W. Levine, B.S.; N. H. Truong B.S.; C. Zaborowski B.S.; M. Thomas, G. Campbell B.S.; D. Decerbo B.S.; D. Williams, C. Ellis B.S.; I. M. Leffak B.S. Ph.D. (Dept. of Biol. Chem. WSU); P. H. Calcott P.I.

Publication: 1, FEMS Letter (Appendix A)

A drug resistance plasmid alters not only the drug resistance of the host strain but also its resistance to freeze-thaw. Plasmid mediated decrease in resistance to freeze-thaw is accompanied by an increased sensitization to EDTA and detergents but not salt (see attached paper). This led us to determine the molecular mechanism of the alteration in cryosensitivity. The plasmid does not alter the phospholipid, neutral lipid or fatty acid composition of the host cell indicating that the lipid portion of the outer and cytoplasmic membranes are unaffected. Since the plasmid altered the organisms sensitivity to detergents but not salt, it would suggest that the cytoplasmic membrane was unaffected by the plasmid while the outer membrane (OM) could be affected. Other than lipid, the OM contains L.P.S. and protein. Analysis of cell L.P.S. contents and purified L.P.S. for chromatographic properties and composition led us to conclude that while small changes in L.P.S. composition were occurring, they were not enough to ascertain the physiological properties observed. Extraction of OM protein by differential solubilization or by ultracentrifugation and analysis by S.D.S.-polyacrylamide gel electrophoresis revealed that the plasmid altered the outer membrane protein composition in a complex fashion, especially in stationary phase organisms.

This system was analyzed further using an outer membrane protein (Palmitoyl CoA esterase) with enzyme activity. We showed that the plasmid altered the susceptibility of the outer membrane to stress. In whole cells, the enzyme is totally cryptic (>99.9%) with 100% activity being expressed after French Press disruption. Partial disruption of the outer membrane by slow or rapid freezing caused partial exposure of the enzyme at the cell surface (enzyme was not released or inactivated by freeze-thaw). In the plasmid-deficient strain, fast freezing exposed less than 20% of the enzyme while slow exposed 83-100% of the activity. Comparable results were obtained in log and stationary phase cultures. Log phase cells of the plasmid containing strain grown in the same medium exposed more of the enzyme after slow and fast freezing, 100 and 65%. Stationary phase organisms did not expose as much of the enzyme even after slow freezing (35%). When the plasmid containing strain was grown in a medium (N.B.) supplemented with mercuric ions and streptomycin, the enzyme was immune to freeze thaw induced exposure (45%). In addition, the presence of the plasmid altered the distribution of protein, L.P.S. (KDO), Palmitoyl CoA

esterase, phosphodiesterase and arabinose binding protein (OM or periplasmic components) but not isocitrate dehydrogenase (an intracellular enzyme) on osmotic shock of the cells. This is further evidence for the plasmid altering the outer membrane and wall. This study will be written up in the next 6 months.

c. Involvement of L.P.S. in cryoresistance

Personnel: G. Bennett B.S.; A. Seaver B.S.; P. H. Calcott P.I.
Publications: 2, ASM Abstract, Applied and Environmental Microbiology paper (Appendix A)

d. Involvement of O.M. proteins in cryoresistance

Personnel: R. Petty B.S. M.S.; K. Calcott, B.S.; M. Thomas;
P. H. Calcott P.I.
Publication: 0.

A series of mutants (ompA, ompB, tax and lpp) of E. coli with altered outer membrane profiles was examined for sensitivity to certain antibiotics, detergents, salt, EDTA and freezing and thawing. While the MIC's for tetracycline, neomycin and polymyxin B were unaffected by the mutations, the MIC's for bacitracin and novobiocin were greatly reduced in some of the mutants. Loss of the ompA or tax gene product did not affect the sensitivity of the organism to challenge with either low or high concentration of SDS in the presence or absence of EDTA. While loss of the ompB gene product protected the organism from these stresses, loss of the lpp gene product severely sensitized them. Loss of any of the gene products reduced the stability of the outer membrane as evidenced by an increased sensitivity of one strain to SDS in the plating medium while very little change in the sensitivity of the strains to NaCl was seen. Loss of the ompA, tax and especially the lpp gene products increased the sensitivity of the strains to slow or rapid freezing and thawing in water or saline while the loss of the ompB gene product protected the organism. Many survivors exhibited both wall and membrane damage, with loss of the tax, ompA or lpp gene products resulting in an increase in the incidence of wall and membrane damage in the survivors while loss of the ompB gene product protected the organism for both types of damage when cells had been subjected to freezing in water. When frozen in saline only loss of the lpp gene product dramatically increased the proportion of survivors with wall and membrane damage. These studies indicate that the outer membrane proteins including the porin proteins (ompA), the structural lipoproteins (lpp) and the structural proteins coded or controlled by ompB and tax play important roles in stabilizing the outer membrane and protecting the organism when stressed.

e. Involvement of lipids in cryoresistance

Personnel: A. Blasingame; T. Grace; P. Bishop; L. Manzo;
K. Dickey; J. N. Oliver (Dept. Biol. Sci., Univ. N. Carolina,
Charlotte); P. H. Calcott P.I.

Publications: 0

A fatty acid auxotroph of E. coli (FabA) is unable to make unsaturated fatty acids at 37°C.³ However, it will incorporate exogenously added unsaturated fatty acids directly into the membrane lipids. Using these techniques, we have determined the effect of chain length and degree of unsaturation of the added fatty acid on the resistance of the organism to freeze-thaw, detergents in resting suspension, and detergents and salt in growing situations. In addition, the effect of added fatty acid on the retention of β -galactosidase (an intracellular enzyme), phosphodiesterase (periplasmic enzyme) and L.P.S. (one component) after freeze-thaw stress was investigated. When organisms were enriched with the physiological fatty acids (16:1 or 18:1) survival from freeze-thaw, retention of enzymes and KDO and resistance to detergents and salt were maximum. When 14:1 or 20:1 was enriched in the membranes, the organism showed more susceptibility to stress. Further sensitivity to the stress was seen for organisms enriched in 22:1 fatty acid. These organisms were so sensitive they released large amounts of KDO and the two enzymes without apparent stress. Enrichment in 18:2 versus 18:1 resulted in a more stable cell to stress with less release of enzyme at KDO, increased resistance to freeze-thaw and increased resistance to detergent and salt.

When E. coli enters stationary phase it modifies its unsaturated fatty acids 16:1 and 18:1 to the appropriate cyclopropane fatty acid ($\Delta 17:0$ and $\Delta 19:0$) by methylating and cyclizing the double ($C=C$) bond. The physiological function of these fatty acids is unknown.⁴ However, some workers have presented evidence to indicate that strains which synthesize high levels are more resistant to freeze-thaw stress than strain which do not. These studies were performed with a highly variable genetic background. Cronan has isolated and characterized two mutants of E. coli (FT16 and 17) which unlike the wild type do not convert unsaturated fatty acid to cyclopropane fatty acid.⁴ Using these strains we have compared log phase (which do not contain the cyclopropane fatty acid) and the stationary phase organisms for resistance to freeze-thaw, detergents in growth and non-growth situations and salt in growing situations as well as release of the enzymes β -galactosidase (internal) and phosphodiesterase (periplasmic) and L.P.S. after freeze-thaw. As shown by Cronan the mutants did not, while the wild-type did, convert unsaturated fatty acids to the cyclopropane form.

Log phase organisms from the three strains showed slight variations in sensitivity to freeze-thaw indicating that the mutations might be altering the cryosensitivity by a mechanism not involving conversion of the unsaturated to cyclopropane fatty acid (no cyclopropane fatty acid was found in any log phase organism). The nature of the mechanism is unknown. However, when stationary phase organisms were examined, it was evident that cryoresistance in water was unaffected by the mutations, while that for organisms frozen in saline was influenced. The resistance for the strain synthesizing no cyclopropane fatty acid (FT16.17) was 2 to 3 fold higher than the strain containing the cyclopropane fatty acid (FT1). This was shown for organisms frozen rapidly or slowly. When stationary phase organisms were compared, survivors of saline freezing in the mutant strains showed less wall and membrane damage than comparable survivors of strain FT1. The difference was less evident for water frozen stationary phase organisms or for water or saline frozen log phase organisms. In addition, sensitivity to detergents during growth and resting states was also influenced by the mutations. While log phase organisms showed comparable resistance to detergents, stationary phase mutant organisms were 3-4 fold more resistant to SLS in growth situations (plating medium) and 2 fold in resting state in the presence or absence of EDTA. Similarly, less phosphodiesterase, and LPS (wall components) and β -galactosidase (a cytoplasmic enzyme) was released from saline frozen stationary phase populations of mutant organisms than the parent wild-type strain; little difference in release was seen from stationary phase organisms frozen in water or from log-phase organisms.

These two studies indicate the role of the lipid components in determining the resistance of organisms to stresses including freezing and thawing.

f. Yeast membrane alterations

Personnel: A. H. Rose B.Sc. Ph.D. (Dept. Biology, University of Bath, England); P. H. Calcott P.I.

Publication: 1, J. General Microbiology, submitted and accepted (Appendix A)

4. Starvation and Substrate-Accelerated Death

Personnel: T. J. Calvert B.S. M.D.; G. Bennett B.S.; M. Thomas; P. H. Calcott, P.I.

Publications: 3, 2 ASM Abstracts, Journal of General Microbiology (Appendix A)

Mr. Calvert investigated the role of cyclic phosphodiesterase (converts cyclic AMP to 5'AMP) in the phenomenon of substrate accelerated death (SAD) in *Klebsiella*. His thesis work was presented at a recent A.S.M. Meeting and published as a paper (J. General Microbiology). This study was continued by G. Bennett (an honors undergraduate) using *E. coli* where genetically defined mutants were available. Using *ova*⁻, (adenylate cyclase⁻, unable to convert ATP to cyclic AMP), *orp*⁻ (catabolite repression protein⁻, unable to utilize internally generated cyclic AMP to induce the *lac*-operon), *cpd*⁻ (cyclic phosphodiesterase⁻, unable to degrade cyclic AMP to 5'AMP) and *orp*⁺ *ova* Δ (deleted adenylate cyclase gene-unable to make cyclic AMP, and a catabolite repression protein that does not require cyclic AMP to function), she determined the critical genes for SAD in *E. coli*. While the wild-type parent showed lactose SAD, the *orp*⁻, *cpd*⁻ and the *ova* Δ *orp*⁺ mutant strains were resistant. The *ova*⁻ strain was sensitive to starvation even in the absence of lactose and was also sensitive in its presence. She deduced that for SAD to occur the cell required active degradation (*cpd*⁺) and synthesis (*ova*⁺) systems as well as a cyclic AMP requiring system (*orp*⁺) in *E. coli*.

In addition, SAD was investigated in *E. coli* further. As reported for *Klebsiella*, lactose SAD caused a release of greater than 99% of the cyclic AMP from the stressed organisms within the first five minutes of stress. Comparable starvation of the organisms in buffer or in a protected environment (Lactose plus 10 mM Mg²⁺) caused less release (~20%) of the nucleotide. Starvation of a wild-type strain (susceptible to SAD) in saline phosphate did not dramatically alter the level of the CRP in the cell whereas starvation in the presence of lactose caused a 3-5 fold increase in the level of the protein. Protection of the organism by cyclic AMP or Mg²⁺ suppressed levels of the protein to those found in buffer starved organisms. As shown for *Klebsiella*, the carbon source in the growth medium played a critical role. Glucose-grown *E. coli* showed glucose SAD but not fructose, mannitol or lactose SAD, while fructose grown cells showed glucose and fructose SAD but not mannitol or lactose SAD. Lactose grown cells showed lactose and glucose SAD and also isopropyl thiogalactoside (IPTG) and thiomethyl galactoside (TMP) (both are inducers of the *lac*-operon and are transported by the *lac* permease yet are not degraded). That the transport mechanism and not metabolism played a critical role in the phenomenon was strengthened since a lactose analogue (thio digalactoside) which binds irreversibly to the *lac* permease and prevents transport, prevented lactose SAD in lactose-grown cells. In addition, a mutant of *E. coli* unable to utilize arabinose (*araA*) was equally sensitive to arabinose SAD when grown in the presence of arabinose as was a wild-type revertant. Permease driven transport of arabinose and not its actual presence in the cell was required for

expression of arabinose SAD since a permease⁻ mutant (still able to transport the sugar by the periplasmic binding protein system) was immune to phenomenon whereas a binding protein⁻ mutant (with active permease) was sensitive. These findings in Klebsiella and E. coli are compatible with SAD being mediated by sugar transport control of the enzyme adenylate cyclase described by Peterkofsky.

Ms. Bennett also investigated the possibility that SAD might involve the unusual nucleotide guanosine tetraphosphate (ppGpp). She found that spoT strains (unable to degrade the nucleotide) were immune to lactose-SAD irrespective of the status of the relA gene (that controls synthesis rate of the nucleotide). This implied that lactose SAD might involve the level of this nucleotide. This was strengthened when ppGpp (1 mM) was shown to protect organisms from lactose SAD.

When organisms are shifted from a complete medium (growth) to a starvation medium (buffered saline), the cell experiences a nitrogen source depletion and stimulates synthesis of ppGpp which accumulates in the cell. However, if they are starved in the presence of lactose, the level of nitrogen source is not only depleted but an unfavorable C/N ratio ensues. The organism responds by synthesizing ppGpp at a faster rate which results in a faster accumulation (2-3 fold). These organisms are susceptible to lactose S.A.D. If the organisms are protected by Mg^{2+} or cyclic AMP, the level of the nucleotide was between 3 and 5 fold higher. Thus depletion of internal cyclic AMP by starvation in the presence of lactose prevented synthesis of ppGpp (external cyclic AMP and Mg^{2+} prevented loss of cyclic AMP from organisms suffering from lactose SAD). The inability to produce internal ppGpp does not seem to be the crucial factor since a relA⁺ spoT strain was unable to synthesize high levels of ppGpp in starvation conditions with lactose but was susceptible to lactose SAD and a relA⁻ spoT⁺ which was unable to synthesize high levels of ppGpp in the presence of lactose and Mg^{2+} was immune. However, when organisms which had been starved in lactose with or without Mg^{2+} were transferred to a growth medium, the intracellular level of ppGpp decreased gradually. In spoT⁺ strains the level of ppGpp was maintained longer in organisms starved in lactose plus Mg^{2+} than lactose alone, irrespective of the state of the relA gene. In spoT⁻ strains, the level of ppGpp was maintained for times comparable to spoT⁺ strains starved in lactose plus Mg^{2+} irrespective of the relA gene state or whether or not Mg^{2+} was included in the starvation environment with lactose.

These studies will be written up as two papers in the forthcoming year and are to be presented at the ASM Annual Meeting, 1982.

5. Cryoprotection

Personnel: A. C. Draper III B.S. M.S.; T. J. Calvert B.S. M.D.;
M. Thomas; P. H. Calcott P.I.

Publications: 2, FEMS letter, Cryoletter (Appendix A)

6. Survival in environmental situations

Personnel: R. Petty B.S. M.S.; P. H. Calcott P.I.

Publication: 0

A variety of methods were evaluated for enumeration of unstressed and freeze-thaw stressed Escherichia coli in a defined laboratory system. All three strains tested, including a laboratory and two natural, isolated strains, behaved similarly. The most probable number method enumerated few of the viable cells while the membrane filter method enumerated higher percentages at both 37°C and 44.5°C; the spread plate method was intermediate. Endo medium enumerated stressed and unstressed cells almost as effectively as nutrient agar at both temperatures while EC and MFC failed to detect a high proportion of the survivors. The stress induced sensitivity to EC and MFC media was repairable, though the kinetics of repair were dependent on temperature of incubation on the viability determination medium and strain of organism. Recovery of organisms inoculated into a frozen soil system in the field was high on Endo medium at 44-5°C, compared with Endo at 37°C, and was also high on MFC at both temperatures only when samples were allowed to repair at 37°C on nutrient agar before assay. Recovery of the stressed organisms on MFC directly was poor.

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APPENDIX A

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THE EFFECT OF FREEZING AND THAWING ON THE TRANSPORT AND RETENTION OF AMINO ACIDS
BY ESCHERICHIA COLI, Susan M. Stuenkel and Peter H. Calcott, Department of Biological Sciences, Wright State University, Dayton, Ohio, 45435.

When E. coli is frozen under either "slow" or "fast" freezing conditions and thawed a proportion of the population fails to divide on a rich medium. Of the survivors, a further portion also fails to divide when stressed with agents such as deoxycholate, sodium chloride and Actinomycin D. These cells are termed structurally injured. Given appropriate conditions, these cells are capable of repairing the damage and regaining the ability to divide on these media. When frozen and thawed, cells energized by various electron-donors partially lose the capacity to transport an amino acid mixture. Stressing agents show the cells to be further damaged when measured by transport capacity. When repaired, cells are capable of transporting the nutrients at rates comparable to that of unfrozen cells. Once the amino acids are transported into the cells (preloaded), they are retained at high levels. Frozen cells lose a proportion of and the ability to retain these compounds especially under stressing situations. Repaired cells transport the released material and retain these nutrients to levels comparable to unfrozen cells. This research was supported by a National Institutes of Health Biomedical Grant to Wright State University and Peter H. Calcott (No. 1-507-RPO-7155-01).

THE EFFECT OF FREEZING AND THAWING ON THE UPTAKE
AND RETENTION OF AMINO ACIDS IN ESCHERICHIA COLI[†]

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FEMS Letters 6:267-272

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[†]Parts of this study have been presented at the American Society for
Microbiology Annual Meeting in Las Vegas, Nevada, U.S.A., May, 1978.

SUMMARY

Escherichia coli is sensitive to freezing and thawing in water or saline when cooled slowly or rapidly. The survival of these populations is dependent on the medium used to assess viability. After freezing and thawing, uninjured, metabolically injured, structurally injured and dead cells can be recognized. Structural damage can be detected in the cytoplasmic membrane and the wall layers. This damage can be repaired given appropriate conditions. Amino acid transport and retention activities which are resistant to cold shock and a detergent, decrease when cells are frozen and thawed. They also become sensitive to the detergent but not to cold shock. Under repair conditions, the activities increase at least partially and regain resistance to the detergent. Statistical analysis indicates that water and saline induced damage during freezing and thawing proceed by different mechanisms with the latter containing most probably, two components, a water induced and a saline induced element.

ABSTRACT SUBMITTED TO:
OHIO ACADEMY OF SCIENCE

PROTEIN SYNTHESIS IN ESCHERICHIA COLI RECOVERING FROM FREEZING AND THAWING DAMAGE.
Baharrudin A. Ghanl and Peter H. Calcott, Dept. of Biol. Sci., Wright State Univ.,
Dayton, Ohio 45435

When bacteria are frozen and thawed, the population loses the ability to form colonies on media particularly those containing selective agents. Surviving populations include injured bacteria with damaged cytoplasmic membranes, DNA and outer membranes. Cells also lose the ability to respire, generate energy and utilize it to drive active transport. We have examined the effect of freeze-thaw on another cell process, protein synthesis. Stressed cells show a dramatic decrease in protein synthesis activity which relates to overall viability but to the level of injury in the population. By comparing the effect of various protein synthesis inhibitors such as chloramphenicol, tetracycline, kasugamycin, streptomycin, puromycin and by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) we have characterized the proteins synthesized immediately after stress. Toluene treatment of E. coli also results in the dramatic decrease in protein synthesis rates with only outer membrane proteins being synthesized. We have characterized the proteins synthesized in this system with the same inhibitors and SDS-PAGE. We have compared the proteins synthesized after toluene treatment and freeze-thaw with those produced by whole cells to determine whether these stresses act differentially on free and membrane-bound ribosomes.

This research was supported by a grant to PHC from U.S. Army Research Office (No. DRXRO-15525-L) and a research fellowship to B.A.G. from M.A.R.A., (Malaysia).

RIFAMPICIN INHIBITS PROTEIN SYNTHESIS IN
TOLUENE-TREATED ESCHERICHIA COLI

BAHARUDDIN A. GHANI AND PETER H. CALCOTT

FEMS Letters (in press)

Rifampicin can inhibit protein synthesis in toluene-treated E.coli by decreasing the half life of messenger RNA. Its mechanism is not by an action on energy generation or RNA synthesis. The use of rifampicin as a specific RNA synthesis inhibitor is questioned.

ABSTRACT

Abdul Ghani, Baharuddin. M.S., Department of Biological Sciences, Wright State University, 1980. Protein Synthesis in Escherichia coli after stress.

When Escherichia coli is frozen and thawed, a portion of the population dies while the survivors are capable of forming colonies. Of the survivors, a portion exhibits membrane damage as evidenced by an inability to form colonies on NaCl-supplemented agar medium while another portion exhibits wall or outer membrane damage since they fail to form colonies on detergent (sodium lauryl sulphate) supplemented agar medium. Yet another portion shows no damage. By manipulating the cooling and warming rates employed to freeze-thaw the population and the freezing menstruum (by including the cryoprotectants glycerol or sucrose), the relative proportions of unharmed, damaged and dead cells can be altered. Protein synthesis is inactivated by freezing and thawing. Similarly by altering the cooling and warming rates and the freezing menstruum, the level of inactivation can be varied. Protein synthesis and viability on nutrient agar, detergent and NaCl-supplemented agar media were positively related with the general formula $y = mx + c$ where $x = \% \text{ protein synthesis remaining}$; $y = \% \text{ viability remaining}$ and m and c being constants. All correlations were significant at the $p < .001$ level.

The proteins synthesized immediately after stress in slow, fast and ultra fast frozen populations were compared with proteins made by unfrozen populations, using sodium-dodecyl-sulphate poly-

acrylamide gel electrophoresis (SDS- PAGE). The patterns of proteins made in unstressed cells were in general different than those produced in stressed cells. The pattern of proteins made in stressed cells were dependent on the cooling rates used to freeze the cells. These patterns were also distinct from those obtained from toluene-treated cells which made only exported proteins. By using ribosome-specific inhibitors (chloramphenicol, puromycin, tetracycline, kasugamycin and streptomycin) and a membrane perturbant (procaine), protein synthesis was characterized in unfrozen, toluene-treated and frozen-thawed cells. In four cases (chloramphenicol, puromycin, tetracycline and procaine), protein synthesis in toluene-treated and unfrozen cells was differentially sensitive to the drug. Frozen-thawed cells showed sensitivity to the drugs comparable to unfrozen and dissimilar to toluene-treated cells. Two drugs (streptomycin and kasugamycin) did not show differences in action on unfrozen, freeze-thawed and toluene-treated cells.

Rifampicin and actinomycin D inhibited RNA synthesis in whole and toluene-treated cells. Protein synthesis in toluene-treated cells was inhibited by rifampicin but not by actinomycin D. Addition of CTP and UTP to the reaction mixture (containing ATP and CTP) did not influence RNA or protein synthesis in toluene-treated cells. Rifampicin but not CTP and UTP, increased the decay of labelled RNA in toluene-treated cells.

From these studies, the following conclusions were drawn.

- 1) Protein synthesis was inactivated by freezing and thawing E. coli. The level of inactivation following stress correlated in a linear $y = x$ manner with the level of injury to the cell membrane in the population.
- 2) The patterns of proteins made after stress, as determined by

the course of this study, rifampicin was found to inhibit protein synthesis in toluene-treated cells. More than 70% inhibition was noticed when rifampicin was added to the reaction mixtures (fig. 22), which already contained ATP and GTP as energy sources. The precise mechanism whereby rifampicin inhibited protein synthesis seemed unclear and could not be resolved with the data available. However, four possible hypotheses could be proposed to explain its action:

- 1) Rifampicin inhibited energy generation
- 2) Rifampicin inhibited RNA synthesis which was a required step in protein synthesis in toluene-treated cells.
- 3) Rifampicin altered the stability of RNA and then inhibited overall protein synthesis.
- 4) Rifampicin inhibited a crucial step in protein synthesis.

Hypothesis 1 seemed unlikely since high levels of ATP and GTP were added as energy source. Hypothesis 2 seemed possible and so the following experiments were undertaken to test it.

If RNA synthesis was important then addition of CTP and UTP together with GTP and ATP might stimulate RNA synthesis and hence protein synthesis in toluene-treated cells. Figure 22 shows that the two nucleotides when included together did not stimulate protein synthesis.

The possibility that RNA synthesis was occurring in toluene-treated cells and whether CTP and UTP stimulated its synthesis were examined. Figure 23 shows that RNA synthesis was occurring in whole cell and was sensitive to rifampicin. RNA synthesis was much less active in toluene-treated cells (4%) and was sensitive to rifampicin. The addition of CTP and UTP did not inhibit or stimulate RNA synthesis.

SDS-PAGE was different, and depended on the cooling rate employed. They were also different from those made by unfrozen and toluene-treated cells.

3) Antibiotic sensitivities of protein synthesis in unfrozen and toluene-treated cells were different for four (chloramphenicol, puromycin, tetracycline, and procaine) out of six antibiotics tested. Sensitivity of protein synthesis by frozen-thawed cells was similar to that of unfrozen and distinct from that of toluene-treated cells.

4) No evidence was obtained to show that membrane-bound and cytoplasmic ribosomes were differentially sensitive to freezing and thawing or that frozen-thawed cells made exported proteins to repair wall and membrane damage exclusively.

5) Rifampicin was shown to increase the degradation of DNA in toluene-treated cells and therefore inhibit protein synthesis in these cells.

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ULTRASTRUCTURAL AND BIOCHEMICAL CHANGES IN ESCHERICHIA COLI INDUCED BY CYTOSKELETAL ACTIVE DRUGS. Peter H. Calcott, David Morton, Din A. Ghani, Rita S. Petty, Micki Thomas and Keith Knisley, Department of Biological Sciences, Wright State University, Dayton, Ohio 45435

Three cytoskeletal active drugs, cytochalasin B, colchicine, and vinblastin, were tested for their ability to disrupt vital processes and ultrastructure in Escherichia coli. To allow entry of the drugs into the otherwise impermeable cells, the bacteria were frozen-thawed in the presence of the drugs. A dose-dependent loss in viability was obtained for each drug after a single freeze-thaw cycle in water but not saline. At concentrations sufficient to kill the bacteria, cytochalasin B, but not colchicine or vinblastin, inhibited DNA synthesis, whereas colchicine and vinblastin, but not cytochalasin B, inhibited protein synthesis directed by cytoplasmic ribosomes. All three drugs inhibited RNA synthesis. Freeze-thawing in water, saline or in the presence of the drugs, caused characteristic disruptions of normal ultrastructure. In general, these treatments produced changes in the granularity of the cytoplasm and distribution of the nucleoid.

These biochemical, ultrastructural and viability data suggest a structural organization for bacterial cytoplasm and nucleoid which is required for basic cell function and growth. It is possible that the cytoplasm structure could be due to the presence of procaryotic equivalents of eucaryotic microtubules, microfilaments and microtrabeculae.

This research was supported by US Army Dept. of Research (Grant No. DRXRO-CB-15525-1).

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INDUCTION OF PLASMID-BORNE MERCURIC ION RESISTANCE
BY STREPTOMYCIN IN PSEUDOMONAS AERUGINOSA

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SUMMARY

Populations of Pseudomonas aeruginosa PA01 harboring a streptomycin and mercuric ion resistance plasmid (pPL1), when not preadapted to growth on mercuric ion media, died or showed extended lags when inoculated into mercuric ion supplemented media. Growth in the presence of mercuric ions or streptomycin caused an adaptation of the population to mercuric ions and eliminated this lag and killing. A NADPH dependent Hg^{2+} oxido reductase was detected in cells grown in the presence of mercuric ions and/or streptomycin which was not present in cells grown in the absence of these agents. Streptomycin acted either as an inducer of the mercuric ion resistance genes or caused duplication of these resistance genes.

Transient Loss of Plasmid-Mediated Mercuric Ion Resistance After Stress in *Pseudomonas Aeruginosa*

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Received 7 April 1980/Accepted 27 March 1981

After freezing and thawing, *Pseudomonas aeruginosa* harboring a drug resistance plasmid (Hg²⁺, Str^r), became acutely sensitive to mercuric ions but not to streptomycin in the plating medium, whereas its sensitivity to both agents became more pronounced indicating a synergistic effect. This freeze-thaw-induced sensitivity was transient and capable of being repaired in a simple salts medium. Transient outer and cytoplasmic membrane damage was also observed in frozen and thawed preparations. From kinetics studies, repair of cytoplasmic membrane damage superseded repair of outer membrane damage and damage measured by mercuric ions and mercuric ions plus streptomycin. Osmotically shocked cells were also sensitive to mercuric ions, mercuric ions plus streptomycin, and sodium lauryl sulfate, but not to sodium chloride or streptomycin alone. This sensitivity was again transient and capable of repair in the same simple salts medium. Active transport of a non-metabolizable amino acid, α -amino isobutyric acid, was sensitive to mercuric ions and became more so after freezing and thawing. A freeze-thaw-resistant mercuric ion-dependent reduced nicotinamide adenine dinucleotide phosphate oxidoreductase was localized in the cytoplasm of this organism. This enzyme and an intact outer membrane appear to be required for mercuric ion resistance in this strain.

TRANSIENT LOSS OF PLASMID MEDIATED MERCURIC ION RESISTANCE
AFTER FREEZING AND THAWING OF PSEUDOMONAS AERUGINOSA

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SUMMARY

After freezing and thawing, Pseudomonas aeruginosa harboring a drug resistance plasmid ($Hg^{2+}R$, Strep^R), became acutely sensitive to mercuric ions but not to streptomycin in the plating medium. Its sensitivity to both agents in the plating medium became more pronounced indicating a synergistic effect of the mercuric ions with streptomycin. This freeze-thaw induced sensitivity was transient and capable of being repaired in a simple salts medium. Transient wall and membrane damage were also observed in frozen-thawed preparations. From kinetics studies, repair of membrane damage superceded repair of wall damage and damage measured by mercuric ions and mercuric ions plus streptomycin. Osmotically shocked cells were also sensitive to mercuric ions, mercuric ions plus streptomycin and sodium lauryl sulfate but not to sodium chloride or streptomycin alone. This sensitivity was again transient and capable of repair in the same simple salts medium. Active transport of a non-metabolisable amino acid, α -amino isobutyric acid (AIB), was sensitive to mercuric ions and became more so after freezing and thawing. A freeze thaw resistant mercuric ion dependent NADPH oxido-reductase was localized in the cytoplasm of this organism. This enzyme and an intact outer membrane appear to be required for mercuric ion resistance in this strain.

Mutagenicity of Freezing and Thawing

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SUMMARY

Freezing and thawing of E. coli in saline but not water at either slow or rapid rates caused mutations in the survivors as evidenced by increase in the frequency of trp^+ revertants from a trp^- auxotroph. Cycles of freeze-thaw, but not frozen storage, augmented the frequency of this mutagenic event in a dose-dependent manner. The relationship was also observed for organisms subjected to ultra-violet light. The cryoprotectant glycerol, not only protected the organisms from loss of viability on freeze-thaw, but also prevented the mutagenic event. Cold, osmotic or cold osmotic shocks did not increase the mutagenic frequency. This indicated that the freezing and/or thawing steps were the responsible events for causing mutation. This suggested that the process of freeze-thaw is mutagenic and that DNA could be a prime target during this stress.

SENSISIVITY OF DNA REPAIR DEFICIENT MUTANTS OF
ESCHERICHIA COLI TO FREEZING AND THAWING

PETER H. CALCOTT AND MICHAL THOMAS

FEMS Letters (In press)

A family of DNA repair deficient mutants of E.coli were assessed for resistance to UV light, X-rays and slow and rapid freezing and thawing. RecA, recB, recC, polA and uvrA mutants were more sensitive to UV light and/or X-rays as shown by others. These same strains were sensitive to both slow and rapid freezing in water and saline, indicating that the gene products played a role in determining cryoresistance. It is proposed that the excision repair and rec-dependant post-replication repair function to repair dna damage introduced into DNA at both slow and rapid cooling rates.

The Role of DNA Repair Genes and an R-Plasmid in
Conferring Cryoresistance to Pseudomonas aeruginosa

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SUMMARY

The resistance of Pseudomonas aeruginosa wild-type, uvr⁻, pol⁻ and rec⁻ mutants to ultra-violet light, x-rays and freezing and thawing was determined. An R-plasmid, pPL1, which increased resistance of the wild-type, uvr⁻, and pol⁻ but not rec⁻ strains to ultra-violet light increased the resistance of only rec⁻ and pol⁻ strains to x-rays and freezing and thawing. These findings reinforce the idea of DNA as a target in the organism for freeze-thaw stress.

THE SENSITIVITY OF DNA REPAIR MUTANTS OF PSEUDOMONAS AERUGINOSA TO FREEZING AND THAWING AND THE ROLE OF A UV REPAIR PLASMID IN CONFERRING RESISTANCE TO STRESS. Donna Williams and Peter H. Calcott, Department of Biological Sciences, Wright State University, Dayton, Ohio 45435

A wild-type (Wt) and rec⁻, pol⁻ and uvr⁻ mutants of Pseudomonas aeruginosa were assessed for sensitivity to UV, X-ray and freeze-thawing. While uvr, pol and rec genes were important in determining sensitivity to UV radiation only pol and rec genes played a role in determining X-ray sensitivity. Freezing and thawing (both slow and rapid freezing, in water and saline) sensitivity was dependent on pol and rec but not uvr gene products. A plasmid (pPL1), which codes for drug resistance, increased survival of wt, uvr⁻ and pol⁻ but not rec⁻ strains towards UV. The plasmid, however, increased survival of rec⁻ and pol⁻ but not wt or uvr⁻ strains when populations were stressed with X-ray or freeze-thaw stress. It is apparent, that freeze-thaw damage could be similar to that produced by X-rays and could be repaired in a similar fashion. This study emphasizes the importance of DNA as a target for freeze-thaw stress. This work was supported by a research grant from the U.S. Army Department of REsearch (No. DRXRO-CB-15525-L).

CURING OF DRUG RESISTANCE PLASMIDS FROM
ESCHERICHIA COLI AND PSEUDOMONAS AERUGINOSA
BY FREEZING AND THAWING

PETER H. CALCOTT, DOUGLAS WOOD AND
LISA ANDERSON
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SUMMARY

A mercuric ion resistance and streptomycin resistance plasmid (pPL1) of Ps. aeruginosa can be cured effectively by heat, sodium dodecyl sulphate and mitomycin C but not by acriflavin or acridine orange. Plasmid RP4 ampicillin resistance, can be cured from E. coli by acridine orange, acriflavin and pyocyanine but not by the other three treatments. If Ps. aeruginosa pPL1 are frozen and thawed either rapidly (100°C/min) or slowly (1-2°C/min) or frozen-stored (at -20°C or -80°C) in 0.85% saline, they are cured of their plasmid to a higher level than unfrozen cells. E. coli RP4 is also cured of its plasmid to an extent significantly above that of unfrozen cells. However, frozen-storage does not cause any more curing than simple freeze-thaw indicating that the freezing and/or thawing component, but not storage, caused lesions in subsequent replication of the plasmid after thawing. It seems likely that freezing and thawing of cells in the environment might play a role in preventing wide spread of plasmids in nature.

DNA DAMAGE AND ITS REPAIR IN FROZEN-THAWED ESCHERICHIA COLI. Jae Chul Song and Peter H. Calcott, Department of Biological Sciences, Wright State University, Dayton, Ohio 45435

When Escherichia coli is frozen and thawed a portion of the population dies while the survivors often show injury. A possible target for this stress is the DNA. We have investigated the integrity of the chromosomal DNA after freezing and thawing in vivo. Both rapid (100°C/min) and slow freezing (1-2°C/min) cause single stranded breaks (SSB) in the DNA of E. coli as detected in ³H-thymidine labelled DNA by alkaline sucrose gradient ultracentrifugation. A relationship was noted between the number of SSB and the viability after stress; however, kinetics were different for the two freezing regimes used. Cryoprotectants (glycerol and sucrose) significantly reduced the number of SSB and protected populations from loss of viability. The DNA damage was effectively repaired in a nutrient rich environment. Inhibitors, operative against repair of UV and X-ray induced damage, inhibited the repair of freeze-thaw induced damage. Mutants defective in recA and polA genes repaired DNA damage less effectively than the wild type, uvrA or lexA mutants. DNA damage, thus, might be an important factor in determining freeze-thaw survival of bacterial cells. This work was supported by a research grant from the U.S. Army Department of Research (No. DRXRO-CB-15525-L).

ABSTRACT

Jae Chul Song, M.S., Department of Biological Sciences,
Wright State University, 1981. DNA Damage and Its Repair
in Frozen-Thawed Escherichia coli.

The effects of freezing and thawing, cycles of freeze-thaw, frozen storage, the absence or presence of cryoprotective agents on population viability and repair of DNA integrity were investigated. DNA integrity was measured by the sedimentation properties of DNA in an alkaline sucrose gradient subjected to ultracentrifugation. DNA damage could be calculated from the sedimentation properties and was expressed as an alteration in sedimentation distance of the molecule from stressed cells compared with unstressed. Relationships between DNA damage and viability were determined and analysed. The conclusions from this study could be summarized:

- 1) DNA is damaged by freezing and thawing in a dose dependent manner (number of cycles and time of frozen storage).
- 2) A dose dependent loss of viability was noted when populations were subjected to frozen storage or cycles of freeze-thaw.
- 3) Cryoprotectants protected cells from loss of

viability on freezing and thawing. These protected cells exhibited less DNA damage than equivalent unprotected ones.

4) Viability after stress correlated significantly with DNA damage. The relationship was different for cryoprotected and unprotected populations. 78% of loss of viability was associated with DNA damage for unprotected population whereas 41% was associated for protected populations.

5) DNA damage introduced by freezing and thawing was capable of repairing in a nutrient rich environment. The repair process was inhibited by hydroxyurea, nalidixic acid, chloramphenicol, arsenate and caffeine.

6) Mutants with defects in DNA repair genes were assessed for sensitivity to ultraviolet light, x-ray and freeze-thaw. All mutants (uvrA, recA, polA and lexA) were more sensitive than the wild type parent to freeze-thaw while their relative sensitive to ultraviolet light and x-ray were similar to that reported by others.

7) The ability of the mutants to repair freeze-thaw induced DNA damage was assessed. All mutants were capable of repairing essentially all DNA damage introduced by the stress. Thus recA, polA, lexA and uvrA genes were not essential for repair of freeze-thaw induced DNA damage.

8) A model was proposed to relate the stress of freeze-thaw to loss of viability and included the role of DNA and membrane damage in the phenomenon.

9) Two models were presented to indicate how freeze-

thaw DNA damage could be repaired in wild type and mutant strains of E. coli.

10) The significance of DNA damage in environmental situations was discussed.

PHENOTYPIC VARIABILITY OF LIPIDS OF ESCHERICHIA COLI
GROWN IN CHEMOSTAT CULTURE

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FEMS Letters (in press)

SUMMARY

Phospholipids and fatty acids were extracted and analysed from Escherichia coli grown in carbon-limited and ammonium-limited basal salts media at 37°C in chemostat at growth rates of 0.1 to 0.55h⁻¹. Phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE), diphosphatidyl glycerol (DPG) and an unknown component were detected in all preparations. Ammonium-limited cells showed a constant composition while carbon-limited cells showed growth rate dependent alterations in the proportions of DPG and PG. At low growth rates the DPG content was low while at high growth rates the proportion increased to that found in ammonium-limited cells. The PG content showed the reverse trend. Fatty acid composition was also modulated by growth conditions. While the overall degree of saturation and average chain length were constant (indicating a constant fluidity of the membrane), the relative proportions of the major fatty acids (C_{14:0}, C_{14:1}, C_{16:0}, C_{16:1}, C_{18:0}, C_{18:1}, C_{18:2}) were dependent on growth conditions. It is postulated that key enzymes in fatty acid and phospholipid biosynthesis are under catabolite repression. Survival of the cells grown under these different conditions appeared to relate to the levels of PG, DPG, and C_{16:0} and C_{18:0} fatty acids in the membrane lipids.

Drug Resistance Plasmid (pPL1) Mediated Changes in
the Susceptibility of Pseudomonas aeruginosa to Stress

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ABSTRACT

A drug resistance plasmid (pPL1) in Pseudomonas aeruginosa increases the strains resistance to U/V light, mercury, gentamycin, streptomycin and freezing and thawing while it decreases its resistance to the presence of surfactants following freezing and thawing and to the presence of EDTA in a non-growth situation. Growth kinetics in the presence of sodium lauryl sulphate but not NaCl was also altered by the plasmid. We conclude that the plasmid alters the chemistry of the wall which changes the susceptibility of the cells to stress.

The Effect of Defined Lipopolysaccharide Core
Defects on Resistance of Salmonella typhimurium
to Freezing and Thawing and Other Stresses.

GEANNIE BENNETT, ALAN SEAVER and PETER H. CALCOTT* Dept.
of Biol. Sci., Wright State Univ., Dayton, OH 45435

A defined set of mutants of Salmonella typhimurium with altered lipopolysaccharide core structure was examined for sensitivity to certain antibiotics, detergents, and freezing and thawing. The MIC's for bacitracin, novobiocin and polymyxin B but not neomycin and tetracycline were greatly reduced in the deep rough mutants. The deepest rough mutants were sensitive to low levels of SDS in starvation conditions; perturbation with EDTA increased sensitivity. High SDS concentrations caused rapid lysis in the presence or absence of EDTA. The rough mutants were more sensitive to freezing, either slowly or rapidly, in water or saline, than the smooth strain. The mutations did not affect sensitivity of the cytoplasmic membrane to freezing and thawing, though the outer membrane was more susceptible to stress. Isolation of crude outer membranes showed that the rough mutants incorporated less protein and more L.P.S. than smooth strains. During the isolation procedure the deep rough mutants released more L.P.S. than the smooth strain. The outer membrane components including L.P.S. and protein are important structurally in determining resistance of Salmonella and possibly other Gram-negatives to stress.

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10 Effect of Defined Lipopolysaccharide Core Defects on
11 Resistance of *Salmonella typhimurium* to Freezing and
12 Thawing and Other Stresses

13 GEANNIE M. BENNETT, ALAN SEAVER, AND PETER H. CALCOTT[†]
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15 Received 21 April 1981/Accepted 16 July 1981

16 A family of mutants of *Salmonella typhimurium* with altered lipopolysaccha-
17 ride (LPS) core chain lengths were assessed for sensitivity to freeze-thaw and
18 other stresses. Deep rough strains with decreased chain length in the LPS core
19 were more susceptible to novobiocin, polymyxin B, bacitracin, and sodium lauryl
20 sulfate during growth, to ethylenediaminetetraacetic acid and sodium lauryl
21 sulfate in resting suspension, and to slow and rapid freeze-thaw in water and
22 saline, and these strains exhibited more outer membrane damage than the wild
23 type or less rough strains. Variations in the LPS chain length did not dramatically
24 affect the sensitivity of the strains to tetracycline, neomycin, or NaCl in growth
25 conditions or the degree of freeze-thaw-induced cytoplasmic membrane damage.
26 The deeper rough isogenic strains incorporated larger quantities of less-stable
27 LPS and (fewer) proteins into the outer membrane than did the wild type or less
28 rough mutants, indicating that the mutations affected outer membrane synthesis
29 or organization or both. Nikaido's model of the role of LPS and protein in
30 determining the resistance of gram-negative bacteria to low-molecular-weight
hydrophobic antibiotics is discussed in relation to the stress of freeze-thaw.

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FREEZE-THAW AND COLD-SHOCK RESISTANCE OF *Saccharomyces cerevisiae*

AS AFFECTED BY PLASMA-MEMBRANE LIPID COMPOSITION

By PETER H. CALCOTT† and ANTHONY H. ROSE*

Zymology Laboratory
School of Biological Sciences
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Bath
Avon
England

PHENOTYPIC VARIABILITY OF LIPIDS OF ESCHERICHIA COLI
GROWN IN CHEMOSTAT CULTURE

Peter H. Calcott and Rita S. Petty
Department of Biological Sciences
Wright State University
Dayton, Ohio 45435

FEMS Letters (in press)

SUMMARY

Phospholipids and fatty acids were extracted and analysed from Escherichia coli grown in carbon-limited and ammonium-limited basal salts media at 37°C in chemostat at growth rates of 0.1 to 0.55h⁻¹. Phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE), diphosphatidyl glycerol (DPG) and an unknown component were detected in all preparations. Ammonium-limited cells showed a constant composition while carbon-limited cells showed growth rate dependent alterations in the proportions of DPG and PG. At low growth rates the DPG content was low while at high growth rates the proportion increased to that found in ammonium-limited cells. The PG content showed the reverse trend. Fatty acid composition was also modulated by growth conditions. While the overall degree of saturation and average chain length were constant (indicating a constant fluidity of the membrane), the relative proportions of the major fatty acids (C_{14:0}, C_{14:1}, C_{16:0}, C_{16:1}, C_{18:0}, C_{18:1}, C_{18:2}) were dependent on growth conditions. It is postulated that key enzymes in fatty acid and phospholipid biosynthesis are under catabolite repression. Survival of the cells grown under these different conditions appeared to relate to the levels of PG, DPG, and C_{16:0} and C_{18:0} fatty acids in the membrane lipids.

ABSTRACT SUBMITTED TO:
AMERICAN SOCIETY FOR MICROBIOLOGY

Summary: Characterisation of cyclic phosphodiesterase in Klebsiella aerogenes and its role in substrate-accelerated death. P.H.CALCOTT* and T.J.CALVERT, Dept. of Biol. Sci., Wright State Univ., Dayton, Ohio, 45435.

Substrate-accelerated death (SAD) is a repression phenomenon whereby the carbon source used for growth or a product of its metabolism reduces the cyclic AMP level in the cells during starvation. This prevents the growth and division of the cells when they are exposed to a complete medium. The cyclic AMP levels in bacteria are governed by adenylate cyclase, cyclic phosphodiesterase and release mechanisms. Cyclic AMP phosphodiesterase in K. aerogenes is a soluble cytoplasmic enzyme which breaks down 3',5' cyclic AMP to 5' AMP with an apparent K_m of 0.3-0.4 mM. It does not show a requirement for metals but is sensitive to EDTA. Sugars and nucleotides do not modulate its activity. Unlike the E.coli enzyme, it is sensitive to theophylline, MIX and caffeine. During starvation of lactose-grown cells in buffer alone, in SAD conditions (lactose, analogs, pyruvate or glucose) or in protected environments (lactose + Mg^{2+} , + theophylline, or + cyclic AMP), the enzyme remains fully active. It is most likely that during SAD, cyclic AMP levels are modulated by adenylate cyclase and that the phosphodiesterase and release mechanisms act as drains on the system to allow rapid turnover of the nucleotide.

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Start→ Involvement of Guanosine Tetraphosphate (ppGpp) in Expression of Substrate-Accelerated Death in Escherichia coli. GEANNIE M. BENNETT and PETER H. CALCOTT,* Dept. of Biol. Sci., Wright State Univ., Dayton, OH 45435 and CR-Bioproducs, Dow Chemical, Midland, MI 48640.

Lactose-grown Escherichia coli is susceptible to lactose-accelerated death (LAD) when starved in saline phosphate supplemented with lactose. However, 0.1 mM ppGpp, 0.1 mM cyclic AMP or 10 mM Mg^{2+} when added exogenously alleviated the phenomenon. When the organism was starved in saline phosphate, it synthesized very low levels of ppGpp, while starvation in the presence of lactose stimulated the synthesis of the nucleotide 2-3 fold. Protected organisms (starved in lactose supplemented with cAMP or Mg^{2+}) synthesized 10-15 fold more of the nucleotide. The presence of a functional spoT gene but not a relA gene was required for expression of LAD. When stressed organisms were added back to growth medium, the level of ppGpp remained higher in those strains resistant to LAD compared with those sensitive. It is proposed that high ppGpp levels are controlled by cyclic AMP and are required by the organism to signal an adverse growth environment (eg. unbalanced C/N ratio) during starvation. Failure to signal the hostile environment results in the failure of the cell control process to coordinate division when an otherwise favorable medium is presented to the organism.

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CHARACTERIZATION OF 3'5' CYCLIC ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE
IN KLEBSIELLA AEROGENES AND ITS ROLE IN SUBSTRATE-ACCELERATED DEATH

PETER H. CALCOTT and THOMAS J. CALVERT
Department of Biological Sciences
Wright State University
Dayton, Ohio 45435

SUMMARY

A cyclic adenosine phosphodiesterase has been detected in Klebsiella aerogenes and characterized in crude extract. It was a soluble cytoplasmic enzyme with apparent K_m of 0.9 mM and pH optimum of 7.0. It was sensitive to EDTA, Mg^{2+} and other metals. The enzyme activity was inhibited or activated by some nucleotides but not by metabolites except pyruvate. It was sensitive to the methyl xanthines, caffeine, theophylline and methyl isobutyl xanthine. During starvation or substrate accelerated death (SAD), the enzyme activity was not modulated. It is postulated that during SAD, the enzyme acts as a drain on the cellular cyclic AMP levels. The cyclic nucleotide levels during SAD are proposed to be modulated directly by adenylate cyclase.

Cryoprotective Action of Non-ionic Detergents on
Bacillus subtilis and Bovine Red Blood Cell

Peter H. Calcott, Thomas J. Calvert, Alfred C. Draper III

F. E. M. S. Letters 4, 1978, 211-215

Summary

Bacillus subtilis can be protected from freeze-thaw damage by two non-ionic detergents, Tween 80 and Triton WR 1339. The protection afforded is dependent on the concentration of detergent and the population density in the freezing menstruum in a similar fashion found previously with Klebsiella aerogenes. Protection by the detergents is afforded almost maximally at ultra-rapid rates (6000°C/min) of cooling while at slow rates (1-2°C/min) no protection is observed; intermediate protection is afforded at rapid rates (100-200°C/min). Membrane damage and its protection has been observed by measuring the release of U/V absorbing material and K^+ from cells. Attempts at protecting bovine red blood cells with these detergents were entirely unsuccessful. The usefulness of these compounds as cryoprotective compounds is minimal.

Protection of Escherichia coli from Slow Freezing and
Thawing by a New Class of Cryoprotectants: Ionophores.

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SUMMARY

EDTA-permeabilized E. coli can be protected from slow freezing and thawing but not rapid freezing and thawing by ionophores such as gramicidin and valinomycin but not by pentachlorophenol. In combination, gramicidin and valinomycin were detrimental to cryosurvival. This implicates the sodium and potassium gradients that accumulate in and around cells during freezing as potential lethal factors to cells subjected to slow but not rapid freezing and thawing. It lends support to Mazur's "Two Factor Hypothesis."